
Book Section:
Woodley, K.T. orcid.org/0000-0003-4002-5176 and Collins, M.O. orcid.org/0000-0002-7656-4975 (2019) Quantitative analysis of protein S-acylation site dynamics using site-specific acyl-biotin exchange (ssABE). In: Evans, C., Wright, P. and Noirel, J., (eds.) Mass Spectrometry of Proteins. Methods in Molecular Biology, 1977 . Humana Press , New York , pp. 71-82. ISBN 9781493992317

https://doi.org/10.1007/978-1-4939-9232-4_6

Quantitative analysis of protein S-acylation site dynamics using site-specific Acyl-Biotin Exchange (ssABE)

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Abstract

Protein S-acylation (palmitoylation) is a reversible lipid modification that is increasingly recognised as an important regulator of protein function including, membrane association, trafficking and subcellular localisation. Most proteomic methods to study palmitoylation allow characterisation of putative palmitoylated proteins but do not permit identification of individual sites of palmitoylation. We have recently adapted the Acyl-Biotin Exchange (ABE) method that is routinely used for palmitoyl-proteome characterisation, to permit global S-acylation site analysis. This site-specific ABE (ssABE) protocol, when combined with SILAC-based quantification, allows both the large-scale identification of palmitoylation sites and quantitative profiling of palmitoylation site changes. This approach enables palmitoylation to be studied at a systems level comparable to other more intensively studied post-translational modifications.

Keywords: Protein, S-acylation, palmitoylation, membrane, proteomic, mass spectrometry, quantitative.

1 Introduction

Protein S-acylation, also known as palmitoylation, is the attachment of a fatty acid to the side chain of a cysteine residue through a thioester bond. It is an important post-translational modification within the cell as it is the only reversible lipid modification and is mainly used as a lipid anchor for membrane attachment (1, 2). The ability to identify S-acylated proteins on a proteome-wide scale has led to the modification being recognised as widespread and involved in a number of processes, with up to 10% of genes having a protein product that is S-acylated (3). There are a number of methods to identify S-acylated proteins which can be divided into three types: removal of the fatty acid and replacement with an affinity tag (often biotin), known as acyl-biotin exchange (ABE) (4), removal of the fatty acid and capture on a thiol-reactive resin (acyl-RAC) (5) or metabolic labelling of S-acylation sites with a clickable fatty acid analogue and click chemistry to replace the fatty acid analogue with an affinity tag, known as metabolic labelling-click chemistry (MLCC) (6). Each method has been shown to enrich an overlapping but distinct subset of the palmitoylated proteome (7), but ABE has the advantage that it can be performed on animal tissues as it requires no previous labelling of palmitoylation sites and the coverage is higher than that obtained using metabolic labelling (7).

One of the major drawbacks of the classical ABE method is that it only provides information of S-acylation on a protein level. We have developed an ABE-based method which allows site-specific analysis of S-acylation, allowing the identification of a large number of S-acylation sites from a single LC-MS/MS run (8). Using this method a significant number of known sites were identified, as well as hundreds of novel sites on known S-acylated proteins, allowing for the first time global analysis of S-acylation site on a proteome scale. This method can be used to probe protein S-acylation in any organism, tissue or cell type and can be combined with a number of quantification types including SILAC (Stable isotope labelling with amino acids in cell culture) to monitor S-acylation site dynamics.

The protocol described here employs SILAC-based quantitation which affords high accuracy quantification to measure small changes in palmitoylation, which is particularly important in perturbation experiments. ssABE may be performed using label-free quantitation (8) to

identify putative sites of palmitoylation and in perturbation experiments where measurement of small changes (less than 2-fold) is not required. A schematic of the workflow for ssABE with SILAC-based quantitation is shown in Figure 1. The workflow for ssABE with label-free quantification is almost identical to ssABE with SILAC-based quantitation, with the exception that samples are not pooled prior to LC-MS/MS analysis; the control and hydroxylamine-treated samples are analysed by two separate LC-MS/MS acquisitions. In perturbation experiments, samples to be compared may be differentially SILAC labelled, pooled in step 1 of the protocol thereby minimising experimental variation (see section 3.8). A detailed description of SILAC-based quantification is not included in this protocol as it is the subject of a dedicated volume of Methods in Molecular Biology entitled "Stable Isotope Labelling by Amino Acids in Cell Culture (SILAC)".

2 Materials

2.1 Protein extraction, reduction and alkylation

1. Extraction buffer: 4 % SDS, 100 mM Tris pH 9, 2 µg/µl aprotinin/leupeptin, 0.5 mM PMSF (see: **Note 1**), 20 µM ZnCl, 5 mM EDTA, 50 mM TCEP, and 25 mM iodoacetamide (prepared fresh).
2. Dounce homogeniser.
3. Heat block/thermomixer.
4. Fine gauge needle and syringe.
5. Tin foil to wrap samples in for alkylation (see **Note 2**).

2.2 Sample clean-up

1. Amicon ultra 30 kD molecular weight cut-off (MWCO) ultrafiltration centrifugal device or similar.
2. 8 M urea/100 mM Tris pH 8: add 100 ml of water to a volumetric flask and add 121.7 g of Trizma base. Add more water up to 800 ml then adjust pH to 8 with hydrochloric acid and make up to 1 L with water to give a 1 M stock solution. Add 500 µl of this stock to a 50 ml tube, weigh out 24 g of urea and add to the tube. Make up to 50 ml

with HPLC grade water (see **Note 3**). Store at room temperature. Prepare urea solution on the day of use.

3. Swinging-bucket centrifuge.

2.3 Acyl-biotin exchange

1. 50 mM Biotin-HPDP: dissolve 4.8 mg biotin-HPDP in 177 µl DMSO. Store 20 µl aliquots at -80°C.
2. N,N-dimethylformamide.
3. 2 M hydroxylamine: Dissolve 1.39 g of hydroxylamine hydrochloride in 5 ml of water. Titrate in 4 M sodium hydroxide until the solution reaches pH 7.4 then make up to 10 ml with water. Prepare immediately prior to use and keep on ice.
4. 8 M urea/100 mM ammonium bicarbonate: Add 5 ml of water to a 50 ml tube, add 24 g of urea and 0.395 g of ammonium bicarbonate and make up to 50 ml with HPLC grade water. Prepare on the day of use and store at room temperature (see **Note 4**).

2.4 Tryptic digestion and peptide collection

1. 100 mM ammonium bicarbonate: Dissolve 0.395 g of ammonium bicarbonate in 50 ml of HPLC grade water. Prepare on the day of use and store at room temperature (see **Note 4**).
2. Trypsin: Re-suspend 100 µg of lyophilised sequencing grade trypsin in 200 µl 0.1 % trifluoroacetic acid. Store aliquots at -20°C.
3. 2 x LB buffer: 100 mM Tris pH 7.4, 300 mM NaCl, 10 mM EDTA, 0.2 % SDS and 0.4% Triton x-100. Store at room temperature.

2.5 Purification of biotinylated peptides

1. Streptavidin agarose resin.
2. 1 x LB buffer: 50 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.1 % SDS and 0.2 % Triton x-100. Store at room temperature.
3. 2 M urea/Tris pH 7.4: add 5ml of HPLC grade water to a 50 ml tube, add 6 g of urea, 500 µl of 1 M Tris pH 7.4 and make up to 50 ml with HPLC grade water (see **Note 3**).
4. 10 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP).
5. 10 % formic acid.
6. SpeedVac or another type of vacuum concentrator.

2.6 LC-MS/MS analysis

1. Formic acid, LC-MS grade.
2. Ultrasonic bath.
3. High-resolution tandem mass spectrometer (e.g. Orbitrap) coupled to a nanoflow chromatography system fitted with a C18 trap column (100 µm, 75 µm x 2 cm, 5 µm) and a 50 cm C18 analytical column (2 µm, 75 µm id).

3 Methods

To be performed at room temperature unless stated.

The following protocol describes the application of ssABE with SILAC-based quantification to measure the enrichment of peptides in hydroxylamine-treated compared to control samples (Figure 1). This quantitative enrichment allows the identification of putative sites of palmitoylation (Figure 2). A variant of this methodology that permits relative quantification of palmitoylation site levels is described in Section 3.7 (Figure 3).

3.1 Protein extraction, reduction and alkylation.

1. Harvest SILAC-labelled (e.g. Lysine-8/Arginine-10) and unlabelled cells (5 x e^6 is usually sufficient) in 1 ml extraction buffer. Homogenise samples 25 times with a Dounce homogenizer (see **Notes 5 & 6**). Transfer the sample to 2ml tubes.
2. Heat at 50 °C for 20 minutes. Allow to cool slightly then shear the DNA by passing the extract through fine gauge needle with a syringe 10 times (see **Note 5**). Hold the needle aperture up against the side of the tube while syringing the sample to make sure the DNA is sheared effectively.
3. Remove any insoluble material by centrifugation at 20,000 g in a benchtop centrifuge for 10 minutes and retain the supernatant.
4. Add additional iodoacetamide to bring the concentration to 50 mM and add urea to a final concentration of 8 M. Incubate the samples covered in tin foil or in the dark (see **Note 2**) for 3 hours at room temperature with rotation to allow alkylation to proceed to completion.

3.2 Sample clean-up.

1. Wash two 30 kDa MWCO spin columns with 8 M Urea/100 mM Tris pH8 and transfer an aliquot (2 mg each) of cell lysate (light and heavy) to each spin column and centrifuge for 10 min at 4000 g to begin buffer exchange.
2. Buffer exchange the samples with the addition of 2 mL 8 M Urea/100mM Tris pH 7.4 and centrifugation for 10 min at 4000 g, repeat five times (see **Note 7**).

3.3 Acyl-biotin exchange.

1. Add 210 µl of 100 mM Tris pH 7.4 to the hydroxylamine-treated (+ HA) sample (heavy SILAC labelled) and 560µl to the control (– HA) sample (unlabelled).
2. Mix 4 µl of 50mM HPDP-biotin with 20µl N, N-dimethylformamide and add to both samples in the upper chamber of the unit to achieve a final concentration of 1 mM biotin.
3. Add 350 µl 2M hydroxylamine (pH 7.4) to the + HA sample to a final concentration of 0.7M. Allow hydroxylamine treatment and biotin labelling of newly released free cysteine residues to proceed for 1 hr at room temperature with gentle agitation.
4. Buffer exchange the samples with the addition of 2 mL 8M Urea/100 mM Tris pH7.4 and centrifugation four times and a final wash with 2 mL 8M Urea/100 mM ammonium bicarbonate.

3.4 Tryptic digestion and peptide collection.

1. Dilute the samples to a final concentration of 1M Urea by the addition of 100mM ammonium bicarbonate. This is most easily achieved by adding 0.5 ml 100 mM ammonium bicarbonate, centrifuging to leave ~0.5ml at 4M urea then topping up to 2 ml with 100 mM ammonium bicarbonate.

2. Digest with sequencing grade trypsin at an enzyme-substrate ratio of 1:50 (40µg trypsin in each sample) for 3 hours at 37 °C with agitation.
3. Collect peptides from the upper chamber, place on ice and wash the chamber membrane with 2 ml 2 X LB buffer.
4. After centrifugation pool the collected peptides (final concentration of 0.5M Urea, 0.1 % SDS, 0.2 % Tx-100, 50 mM Tris pH 7.4, 150 mM NaCl, 50 mM ammonium bicarbonate) and store overnight at -20°C (see **Note 8**).

3.5 Purification of biotinylated peptides.

1. Wash 100 µl bed volume of Streptavidin-agarose resin 3 times with 1 ml of 1 x LB buffer, add to the pooled peptide samples (pooled Light (control) and heavy (hydroxylamine-treated) and incubate for 1 hr at room temp.
2. Collect the beads by gentle centrifugation and wash the resin with 10 ml of 1 x LB buffer, incubating for 10 minutes with gentle agitation and repeat.
3. Transfer the resin to a mini-column and wash twice with 10 ml wash buffer (2 M Urea/100 mM Tris pH 7.4) using a syringe, then with 2 ml HPLC grade water.
4. Elute peptides by the addition of 50 µl of 10 mM TCEP and incubate for 10 min at 37°C. Repeat this step, pool the elutions and acidify by the addition of 10% formic acid (see **Note 9**).
5. Dry the peptides down in a speed vac (see **Note 11**). Peptides can be stored at -20°c

3.6 LC-MS/MS analysis.

1. Re-suspend dried peptides in 40 µl of 0.5 % formic acid by pipetting up and down a number of times. Place samples in an ultrasonic bath for 5 minutes to ensure full resuspension.
2. Centrifuge samples at 20,000 g for 5 minutes and transfer the supernatant (see **Note 12**) to an autosampler vial.

3. Samples are now ready for LC-MS/MS analysis. Ensure that the system is calibrated and quality control checks are performed e.g. by analysis of a standard protein digest.
4. Peptides are desalted on a capillary trap column for 10 min at a flow rate of 5 µl/min and then separated using 120 min reverse phase gradient of 5 to 35 % acetonitrile on the analytical column with a flow rate of 0.25 µl/min.
5. For a data dependent analysis the mass spectrometer may be operated with a cycle of one MS acquired at high resolution e.g. (60,000 at m/z 400), with the top 15 most abundant multiply-charged (2+ and higher) ions in a given chromatographic window subjected to fragmentation (e.g. collision induced dissociation, CID). See **Note 13**.

3.7 Mass Spectrometry data analysis.

1. Process raw MS data files using the data analysis platform MaxQuant (9) or equivalent, searching the data against an appropriate up to date species-specific UniProt sequence database.
2. Set up the search using the following parameters: trypsin as the digesting enzyme with a maximum of 2 missed cleavages, 7 ppm for MS mass tolerance, 0.5 Da for MS/MS mass tolerance (these settings are mass spectrometer dependent), with acetylation (Protein N-term) and oxidation (M) as variable modifications and carbamidomethyl (C) as a fixed modification.
3. Palmitoylation sites are reported and localised in peptide sequences by the use of a “loss” of 57.02146 Da (i.e. no carbamidomethyl modification/unmodified cysteine) as a variable modification (see **Note 14**) as only those cysteines targeted by acyl-biotin exchange should be unmodified, all other cysteines should be alkylated.
4. A protein False Discovery Rate (FDR) of 0.01 and a peptide FDR of 0.01 should be used for identification level cut-offs.
5. Load the “Palmitoylation sites” txt file generated by MaxQuant into Perseus.
6. Remove “reverse” and “contaminant” identifications.
7. In order to identify confidently assigned sites of palmitoylation in peptide sequences, remove those identifications with a localisation score of < 0.75 and a score diff <5.

8. Filter the data so that only putative palmitoylation site identifications with at least 3 valid values (i.e. 3 measured peptide ratios) across biological replicates in the hydroxylamine treated samples and log2 transform these values.
9. Check that the correlation of peptide ratios between replicates is high and that the data is normally distributed.
10. Peptide ratios calculated by MaxQuant are then used to quantify enrichment of peptides in +/- hydroxylamine treated samples across replicates. A minimum of 3 biological replicates is required.
11. Perform a one-sample test with a Benjamini-Hochberg FDR calculation in Perseus. Peptides within an FDR of 0.05 and an S0 value =1 are statistically enriched in the hydroxylamine treated versus control.
12. In order to increase the stringency of analysis and additional fold change enrichment filter should be applied (at least a 3 fold change).
13. The resultant set of sites is defined as being quantitatively enriched in the hydroxylamine treated samples and therefore peptides with putative sites of palmitoylation. See **Notes 15** and **16** for identification of false positives.
14. Further validation of sites can then be performed using techniques such as site-directed mutagenesis and PEG switch methods (10).

3.8 Alternative workflow for relative quantification of S-acylation sites in perturbation experiments.

Once the repertoire of palmitoylation sites expressed in the system of interest has been characterised using the standard ssABE protocol described above, a variation of this procedure may be used to monitor changes in palmitoylation levels in perturbation experiments.

1. In order to accurately compare palmitoylation levels between control (untreated) and treated conditions, 2-plex (1 treatment condition) or 3-plex (2 treatment conditions) cell lysates from SILAC labelled cells are pooled at the beginning of the modified ssABE protocol (Figure 3).

2. The pooled sample is reduced/alkylated and treated with hydroxylamine and processed and analysed as a single sample. This allows for precise quantitation of palmitoylation levels between conditions.
3. If the treatment duration is likely to cause changes in protein expression (e.g. gene knockout, knockdown or chronic drug treatment) then global protein expression profiling of the systems will also need to be performed.
4. Palmitoylation site levels may then be normalised to protein expression levels to reflect true changes in palmitoylation. However, if the treatment duration is acute then expression profiling is not required.

4 Notes

1. Instead of PMSF, leupeptin and aprotinin, a protease inhibitor cocktail can be used at appropriate dilutions.
2. Samples need to be wrapped in tin foil to protect them from light as iodoacetamide is photosensitive.
3. Solvation of urea is an endothermic process, so it can take some time. Placing the tube in a warm water bath (< 37 ^{o}C) will speed up the process.
4. Ammonium bicarbonate needs to be prepared fresh as it breaks down leading to a loss of buffering capacity of the solution.
5. To avoid bubbles/foam in the samples, the plunger of the homogeniser should stay below the level of the liquid. Similarly, excessive foaming can occur during sheering of DNA if the entire sample is drawn up into the syringe, so it is best to leave some of the sample in the tube when sheering DNA.
6. The sample will be very viscous after homogenisation due to the DNA content so it can be difficult to pipette during transfer to the tubes.
7. Volume after each centrifugation step should be around 0.5 ml in the upper chamber. It can take more than one centrifugation step to fully remove all the liquid in the upper chamber to achieve a 0.5 ml volume.
8. An aliquot (20 µl) of the sample can be taken at this point for LC-MS/MS analysis to ensure the digestion step has been successful.

9. Ensure all solutions used in this step are made up with HPLC grade water to prevent any potential interference in the LC system by contaminants.
10. The collection tube which the filter unit fits into will need to be changed multiple times to avoid the waste liquid that has passed through it contaminating the filter unit during the biotinylation and digestion steps. The unit fits well into a standard 50ml falcon tube.
11. Do not to over-dry the peptides as it can be difficult to re-suspend them prior to analysis.
12. It is important to avoid the transfer of any of the pellet at this point as any insoluble material could block either the autosampler needle or the HPLC trap column.
13. Acquisition parameters should be adjusted according to the type of instrument used. MS scans should be acquired with high resolution for SILAC-based quantification.
14. An “unmodified cysteine” modification is not available as standard in the MaxQuant package but can be added in the Andromeda configuration by specifying a modification with the following composition: H(-3) O(-1) C(-2) N(-1) (Δ -57.02146 Daltons) and a cysteine specificity.
15. A number of known false positives can be found as positive hits for palmitoylation during the downstream analysis. A major source of false positives can arise from incomplete alkylation of cysteine residues; the extent of alkylation should be checked. In addition, enzymes which bind biotin and enzymes which use thioester intermediates in their reaction mechanism are known false positives. Such proteins will need to be removed from the dataset before further data analysis.
16. It is important to cross-reference any putative sites of palmitoylation with known disulphide bonds. While it is not impossible that a cysteine residue could be palmitoylated and involved in a disulphide bond depending on the context, there is no way of knowing if this is the case or the positive hit is due to incomplete disulphide reduction and blocking of the resulting cysteine.

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Figure 1

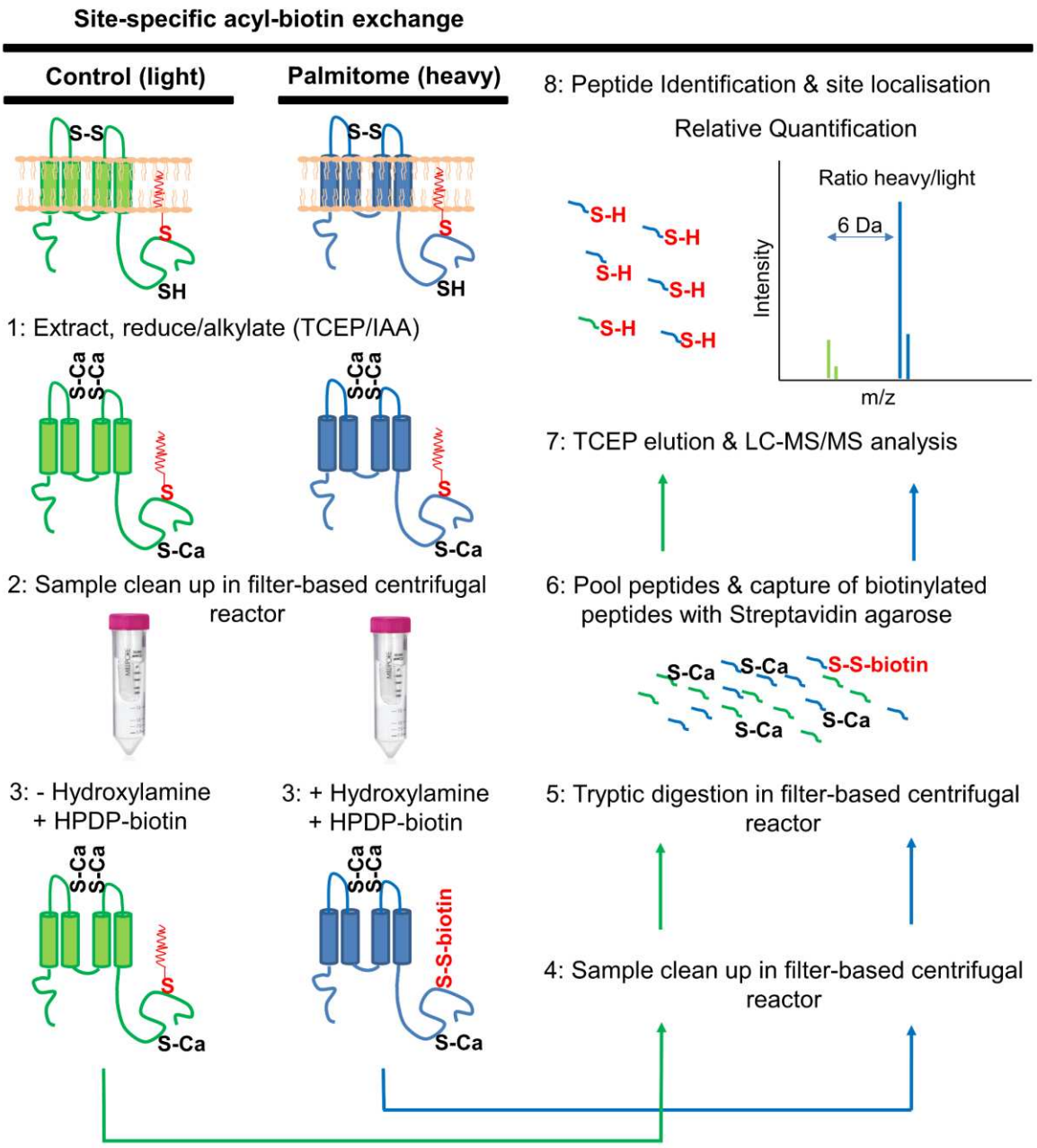
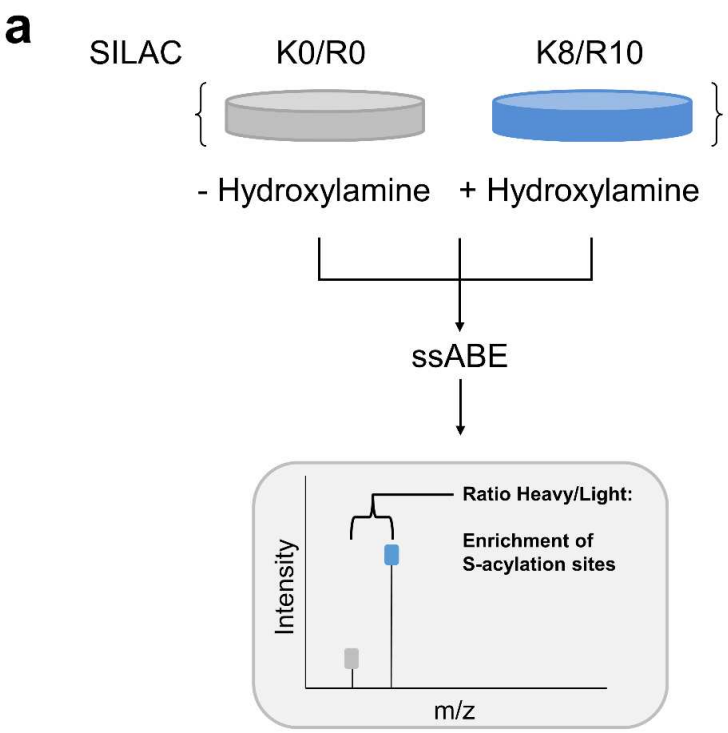


Figure 1. Schematic representation of the ssABE workflow. Protein samples are (1) reduced and alkylated and transferred to a filter-based centrifugal unit in which the following steps are performed; (2) rapid removal of reagents, (3) release of palmitoyl groups from proteins by hydroxylamine treatment and concomitant biotinylation of these newly "free" cysteine residues with a TCEP-cleavable biotin (HPDP-biotin) (4) rapid removal of reagents, followed

by (5) digestion of proteins into peptides and pooling of heavy and light peptides. (6) Biotinylated peptides are captured on Streptavidin agarose beads, washed and eluted (using TCEP) as free cysteine-containing peptides and (7) identified and quantified by LC-MS/MS analysis (8). Quantitative enrichment of peptides in Palmitome (plus hydroxylamine, (heavy)) versus Control (minus hydroxylamine, (light)) samples allows identification of previously palmitoylated peptides. Palmitoylation sites are localised in peptide sequences by the presence of non-alkylated (free) cysteine residues.

Figure 2



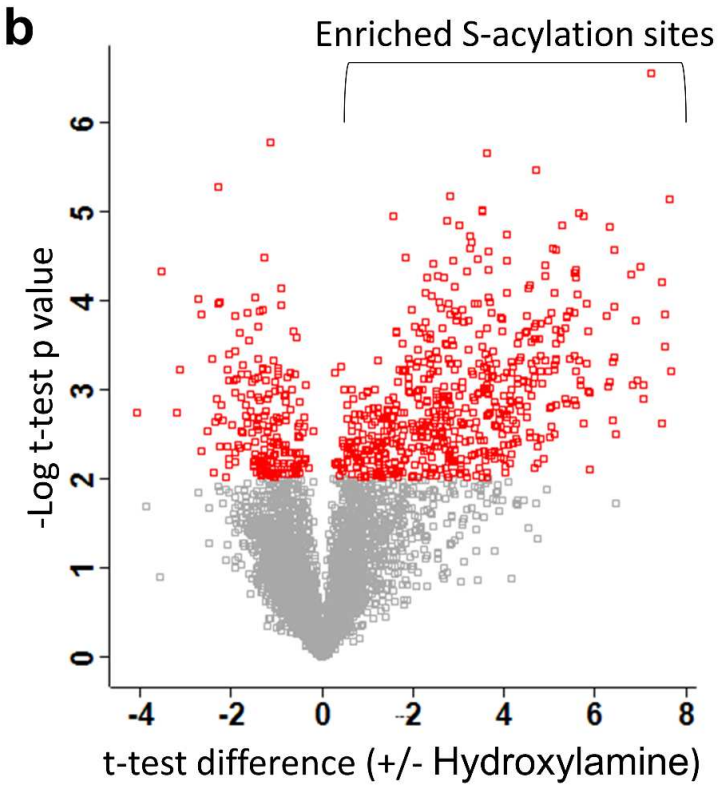


Figure 2. Identification of putative S-acylation sites using ssABE and SILAC-based quantification. (a) A 2-plex SILAC experiment is performed where light (unlabelled) cells (-hydroxylamine) and heavy labelled cells (K8/R10 (Lysine 8/Arginine 10) (+hydroxylamine) are used for a ssABE workflow to identify S-acylation sites. **(b)** Volcano plot showing the quantitative enrichment of peptides in plus versus minus hydroxylamine treated samples. Statistically significant hits (using t-testing and a permutation-based FDR) from this analysis are putative S-acylation sites.

Figure 3

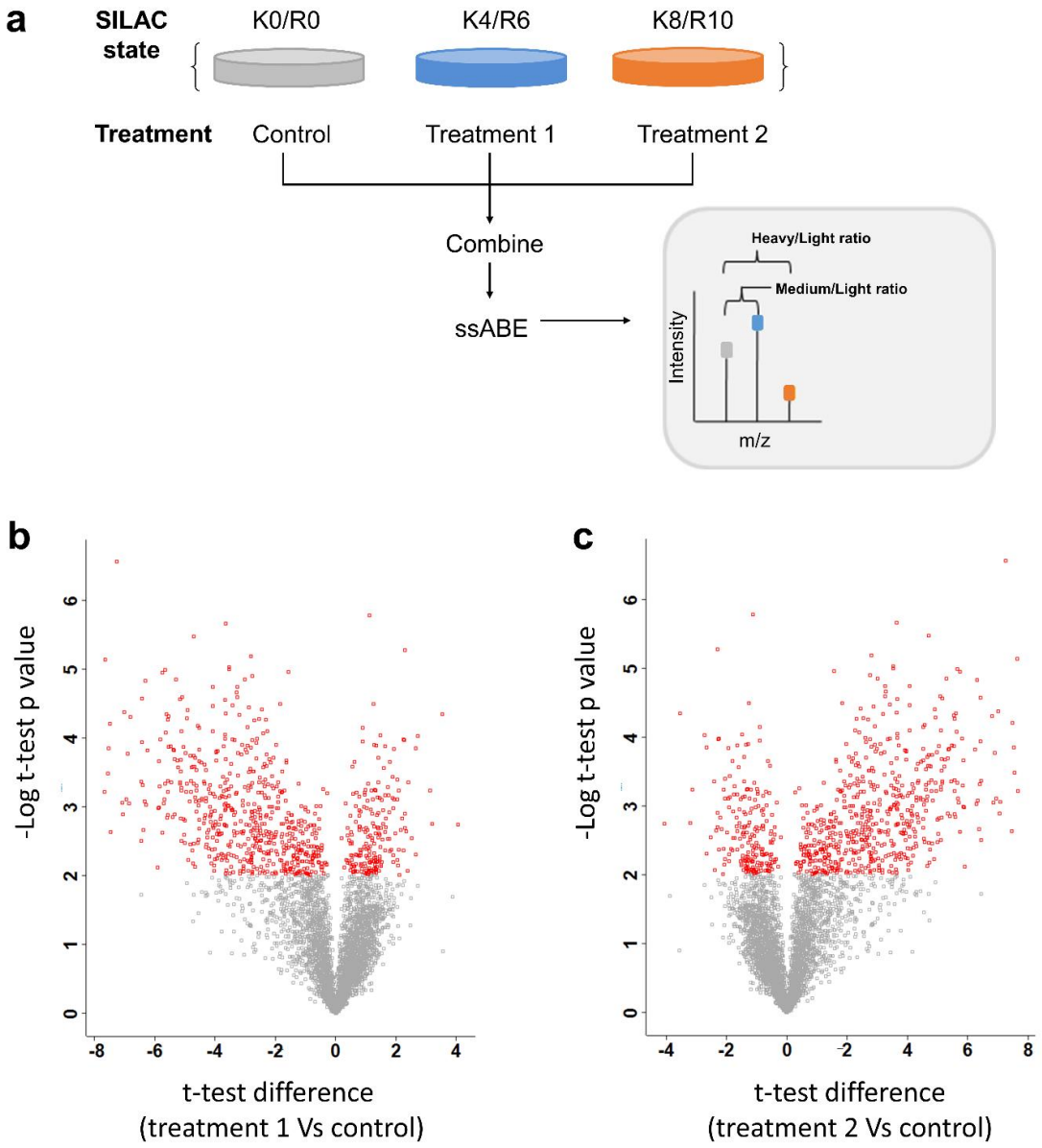


Figure 3. Quantitative assay of global S-acylation site changes in perturbation experiments. **(a)** Schematic representation of a 3-plex SILAC experiment in which cells are either mock treated (light, K0/R0), or undergo two treatment/perturbation conditions (medium, K4/R8 and heavy K8/R10). Cell lysates are combined and are processed using the ssABE workflow with hydroxylamine treatment. **(b-c)** Volcano plots showing the quantitative differences between treatment 1 and control and treatment 2 and control. Differentially regulated S-acylation sites are identified using t-testing and a permutation-based FDR.